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Original article

Arginine vasopressin and its analogues – The influence of position 2 modification with 3,3-diphenylalanine enantiomers. Highly potent V_2 agonists

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ABSTRACT

Eleven new analogues of arginine vasopressin (AVP) modified in position 2 by 3,3-diphenyl-L-alanine or its D-enantiomer (Dip or D-Dip) were synthesized and pharmacologically evaluated for their pressor, antidiuretic and in vitro uterotonic activities. Both the Dip and D-Dip modifications at position 2 of AVP are sufficient to completely change the pharmacological profile of the peptides. They preserve or increase antidiuretic activity, cause its prolongation, transform uterotonic property in antagonistic one and cancel the effect on blood pressure. Four of the new peptides ([Mpa^1,D-Dip^2]AVP, [Mpa^1,D-Dip^2,Val^4]AVP, [Mpa^1,D-Dip^2,D-Arg^8]VP, [Mpa^1,D-Dip^2,Val^4,D-Arg^8]VP) are exceptionally potent antidiuretic agents with significantly prolonged activities.

1. Introduction

Arginine vasopressin (AVP), a neurohypophyseal hormone and neuromodulator, is a cyclic nonapeptide with a disulfide bridge between Cys residues at positions 1 and 6 [1]. This results in a six-amino-acid cyclic part and a C-terminal amidated three-residue tail.

Main physiological roles played by AVP are the regulation of water balance, the control of blood pressure, and the secretion of adrenocorticotropin hormone (ACTH) [1–3]. Moreover, AVP also exhibits to some extent typical oxytocin (OT, a closely related neurohypophyseal peptide) activities such as the galactogogic and the uterotonic effects [4]. Apart from these well-known functions, AVP is also involved in glycogenolysis in the liver [5], in the steroid and catecholamine secretion by the adrenals [6], release of insulin and glucagon by the pancreas [7], and in the secretion of atrial natriuretic factor from the heart [8]. Vasopressin exerts its biological functions upon binding to four different 7-transmembrane G-protein-coupled receptors (GPCRs) termed: V_{1a}, V_{1b}, V_2 and OT receptors [1–3]. V_{1a} receptors, present in many tissues including brain, mediate the vasopressor actions of AVP [1]. V_{1b} receptors present in the pituitary, pancreas and adrenals mediate the ACTH-release from the anterior pituitary gland [3], steroid secretion from the adrenal medulla [6] and the insulin and glucagon release from the pancreas [7]. OT receptors control milk ejection and uterine smooth muscle contractions. These three receptor subtypes function *via* the phosphatidylinositol pathway. The antidiuretic activity of AVP is evoked by V_2 receptors present in the renal tubule and particularly the collecting ducts. They are linked to adenylate cyclase signaling [3].

Vasopressin plays an important role in water metabolism and impairment of its synthesis, secretion or metabolism induces some clinical disorders such as inappropriate antidiuretic hormone secretion syndrome (SIADH) or diabetes insipidus. Elevated AVP secretion leads to renal water retention and extracellular fluid expansion that is compensated for by enhanced urinary Na^+ excretion. The combination of water retention and Na^+ excretion leads to hyponatremia. Criteria for SIADH were first described by Bartter and Schwartz [9] and are still applicable today. These are: hypotonic hyponatremia, urine osmolality higher than appropriate

Abbreviations: Acc, 1-Aminocyclohexane-1-carboxylic acid; Aic, 2-Amino-indane-2-carboxylic acid; Apc, 1-Aminocyclopentane-1-carboxylic acid; AVP, Arginine vasopressin; Cpa, 1-Mercaptocyclohexaneacetic acid; Dip, 3,3-Diphenyl-L-alanine; D-Dip, 3,3-Diphenyl-D-alanine; MBHA, *p*-Methoxybenzhydrylamine; Mob, 4-Methoxybenzyl; NMP, 1-Methyl-2-pyrrolidone; OT, Oxytocin.

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for the concomitant plasma osmolality, increased natriuresis, absence of edema or volume deletion, and normal renal and adrenal functions [10]. On the other hand, diabetes insipidus is a heterogeneous condition characterized by polyuria and polydipsia caused by lack of vasopressin secretion (physiological suppression following excessive water intake) or kidney resistance to its action [11].

Since 1954, when du Vigneaud et al. characterized and synthesized AVP [12], many analogues of this hormone have been obtained and pharmacologically evaluated. A synthetic vasopressin analogue, V_2 agonist, 1-deamino-8-D-arginine vasopressin (dDAVP, commercial names Minirin, Minrin, Desmopressin, Adiuretin-SD) has been a standard drug for the treatment of diabetes insipidus for over 30 years [13]. On the other hand, V_2 antagonists have potential therapeutic value in the treatment of SIADH [14]. The OT antagonists have been useful for suppressing premature labor [15]. The design of analogues that selectively interact with appropriate AVP receptors is still a matter of interest.

Parallel to the development of selective peptide agonist and antagonists, the research of non-peptide agonists and antagonists was carried out. As a result, a number of interesting compounds of varied structure have been synthesized and tested for their activity. At present there are available non-peptide antagonists for all 4 types of neurohypophyseal hormone receptors [16,17], e.g. orally active V_2 receptor antagonists (Vaptans), notably, Tolvaptan, Lixivaptan and Satavaptan [16] and the mixed V_{1a}/V_2 receptor antagonist Conivaptan (YM087) [18]. To date, only Conivaptan (also known as Vaprisol) has been approved by the US FDA for clinical use (by i.v. administration) for the treatment of euvolemic and hypervolemic hyponatremia in hospitalized patients [16]. Especially interesting is the recent development of orally active specific V_{1b} receptor antagonist (SSR149415) which opened new possibilities for the study and treatment of stress and depression [19].

Biological activity of peptides is determined by their structure and conformation. Conformational restriction is therefore a well-established strategy to change their pharmacological profile. Peptide flexibility can be restricted by a local constraint imposed, e.g. by introducing amino acids with limited conformational freedom that has an impact on specific orientations of the other amino acid side chains and peptide backbone.

It is generally accepted that the conformation of the N-terminal part of neurohypophyseal hormone analogues is crucial for their pharmacological activity [20,21]. This has also been supported by our already 12-years research focused on the impact of steric restriction and bulky substituent in the N-terminal part of AVP molecule on biological properties of the resulting analogues. We demonstrated that the arginine vasopressin analogues modified at position 2 with either L or D β-(1-naphthyl)-alanine were moderately potent and selective oxytocin antagonists in vitro [22], while [D-Arg^8]VP substituted at position 3 with β-(2-naphthyl)-alanine turned out to be a potent and selective antagonist of the pressor response to AVP [23]. We have also shown that introduction of either 1-aminocyclohexane-1-carboxylic acid (Acc, also known as Ac_6c) or 1-aminocyclopentane-1-carboxylic acid (Apc, also known as Ac_5c) into position 2 of AVP and some of its analogues resulted in compounds having high antidiuretic potency, low and graded pressor activity, and either no activity or low oxytocin antagonizing activity in the uterotonic in vitro tests [24–26]. Recently, we described some pharmacological activities of three analogues having bulky 3,3-diphenyl-L-alanine (Dip) in position 2 [27]. The new peptides had strikingly different biological properties in comparison with those of the parent hormone. Two of them, [Dip^2]AVP and [Mpa^1,Dip^2]AVP, displayed no pressor activity while their antidiuretic potency was preserved and prolonged (a steep dose–response curve). Both were moderately potent blockers of the oxytocin uterotonic activity.

In this study we further checked the influence of the bulky Dip isomers (L- and D, see Fig. 1) at position 2 of the AVP and some of its analogues on the pharmacological properties. The synthesized analogues **I–XI** are listed in Table 1.

2. Chemistry

2.1. General

Thin-layer chromatography (TLC) was carried out on silica plates (Merck), and spots were visualized with iodine. The solvent system used was butan-1-ol/acetic acid/water/ethyl acetate (1:1:1:1, v/v). High-performance liquid chromatography was carried out on a Waters (analytical and preparative) chromatograph equipped with a UV detector ($\lambda = 226$ nm). The purity of the peptides was determined on Vydac C_{18} column (5 μm, 4.6 × 250 mm) or Hypersil ODS C_{18} column (5 μm, 4.6 × 250 mm) or Hypersil BDS C_{18} column (5 μm, 4.6 × 250 mm). The following solvent systems were used: [A] 0.1% aqueous TFA, [B] acetonitrile/0.1% aqueous TFA (80:20 v/v). Preparative HPLC was carried out using a Waters C_{18} column (15 μm, 100 Å, 7.8 × 300 mm). The mass spectra of the peptides were recorded on a MALDI TOF mass spectrometer.

Mpa(Mob) was obtained as described for Mpa(Bzl) [28] using *p*-methoxybenzyl chloride. Cpa(Mob) was synthesized using a procedure described in literature [29].

All the amino acid derivatives were purchased from Nova-Biochem, except Boc-Dip and Boc-D-Dip, which were provided by ChemImpex.

2.2. Peptide synthesis and purification

All peptides were obtained manually using Boc-chemistry on methoxybenzhydryl resin (MBHA resin, Senn Chemicals AG, 1% DVB, 200–400 mesh, 0.67 mmol/g) on scale of 150 μmol according to standard procedures, using in situ neutralization [30]. Fully protected peptide resin was synthesized according to standard procedures involving (i) deprotection steps 33% TFA/DCM in the presence of anisole (1%), 5 and 10 min; (ii) neutralization with 10% TEA/DCM, 5 and 10 min; (iii) couplings of Boc-amino acid; 3 h at room temperature. In most cases, the amino acids were coupled at 3-fold excess using TBTU/HOBt/NMM in the mixture of DMF/NMP (1:1 v/v) containing 1% Triton, and if necessary amino acid/HATU/HOAt/NMM (1:1:1:2) in mixture of DMF/NMP (1:1 v/v) containing 1% Triton for recoupling steps. After 3 h coupling time, the Kaiser test [31] or chloranil test [32] was performed to estimate the completeness of the reaction. After completion of the synthesis, the protected peptidyl or acylpeptidyl resins were treated with 10 mL of liquid hydrogen fluoride (HF) containing 1 mL of anisole at −70 °C and stirred for 60 min at 0 °C [33]. After removal of HF and

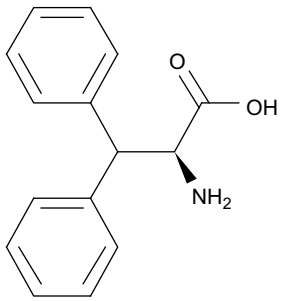

Fig. 1. Structure of 3,3-diphenylalanine.

Table 1
Physicochemical properties of peptides **I**–**XI**.

Analogue		Formula	HPLC T_R (min)		$[M+H^+]$	
			a[a]	b[b]	Calculated	Found
[Mpa^1,Dip^2,Val^4]AVP	**I**	$C_{52}H_{69}N_{13}O_{10}S_2$	15.85	19.06[c]	1100.3	1100.5
[Dip^2,D-Arg^8]VP	**II**	$C_{52}H_{69}N_{15}O_{11}S_2$	11.41	15.62	1144.3	1144.3
[Mpa^1,Dip^2,D-Arg^8]VP	**III**	$C_{52}H_{68}N_{14}O_{11}S_2$	14.19	17.99	1128.5	1129.2
[Mpa^1,Dip^2,Val^4,DD-Arg^8]VP	**IV**	$C_{52}H_{69}N_{13}O_{10}S_2$	40.06[d]	18.68	1100.3	1100.2
[D-Dip^2]AVP	**V**	$C_{52}H_{69}N_{15}O_{11}S_2$	12.74	14.49[c]	1144.3	1144.2
[Mpa^1,D-Dip^2]AVP	**VI**	$C_{52}H_{68}N_{14}O_{11}S_2$	14.46	18.21	1129.3	1129.2
[Cpa^1,D-Dip^2]AVP	**VII**	$C_{57}H_{76}N_{14}O_{11}S_2$	16.16	21.13	1197.4	1197.4
[Mpa^1,D-Dip^2,Val^4]AVP	**VIII**	$C_{52}H_{69}N_{13}O_{10}S_2$	16.52	22.08	1100.3	1100.2
[D-Dip^2,D-Arg^8]VP	**IX**	$C_{52}H_{69}N_{15}O_{11}S_2$	12.95	14.74[c]	1144.3	1144.2
[Mpa^1,D-Dip^2,D-Arg^8]VP	**X**	$C_{52}H_{68}N_{14}O_{11}S_2$	14.80	18.49	1129.3	1129.5
[Mpa^1,D-Dip^2,Val^4,D-Arg^8]VP	**XI**	$C_{52}H_{69}N_{13}O_{10}S_2$	16.64	22.45	1100.3	1100.3

[a] Linear gradient from 20 to 80% of [B] in [A] for 20 min, Vydac C_{18} column.
[b] Linear gradient from 30 to 90% of [B] in [A] for 30 min, Hypersil ODS C_{18} column.
[c] Linear gradient from 30 to 90% of [B] in [A] for 30 min, Hypersil BDS C_{18} column.
[d] Linear gradient from 1 to 60% of [B] in [A] for 60 min, Vydac C_{18} column; [A] 0.1% aqueous trifluoroacetic acid (TFA), [B] acetonitrile: 0.1% aqueous TFA (80:20 v/v).

anisole in vacuo, the mixture was washed successively with anhydrous diethyl ether, then with acetic acid and the solution was diluted with methanol. The resulting dithiols were oxidatively cyclized with a 0.1 M I_2 in methanol using a standard procedure [34]. The solvents were evaporated under reduced pressure and the residue was dissolved in water and lyophilized.

All the products were purified by gel filtration chromatography on Sephadex G-15 using 30% acetic acid as the eluent. After freeze-drying, the fractions comprising the major peak were finally purified by RP-HPLC Waters chromatograph equipped with a UV detector ($\lambda = 226$ nm), Waters C_{18} column (15 μm, 100 Å, 7.8 × 300 mm) in the linear gradient running from 25 to 55% of [B] for 90 min for analogues **I**, **III**, **IV**, **VI**–**VII**, **X**, **XI**, and from 15 to 45% for 90 min for analogues **II**, **V**, **IX**, at a flow rate of 2.5 mL/min; [A] 0.1% aqueous trifluoroacetic acid (TFA), [B] acetonitrile/0.1% aqueous TFA (80:20, v/v). The appropriate fractions were pooled and lyophilized. The analytical HPLC (Waters, equipped with Vydac C_{18} column (5 μm, 4.6 × 250 mm) or Hypersil ODS C_{18} column (5 μm, 4.6 × 250 mm) or Hypersil BDS C_{18} column (5 μm, 4.6 × 250 mm)) produced single peaks with at least 98% of the total peptide peak integrals. The final verification of the peptide sequence and purity were achieved by MALDI TOF mass spectroscopy (we did not detect unexpected molecular ions). The physicochemical properties of new analogues are summarized in Table 1.

3. Pharmacology

3.1. Bioassay methods

Wistar rats were used in all experiments. Handling of the experimental animals was done under supervision of the Ethics Committee of the Academy of Sciences according to § 23 of the Law of the Czech Republic No. 246/1992.

3.2. Uterotonic activity

The uterotonic test was carried out in vitro on the strips of rat uterus in the absence of magnesium ions [35,36]. Rats in induced estrus by the injection of estrogen 48 h before the experiments were used. After decapitation, the uterine horns were excised, longitudinally cut, placed into a bathing chamber and hooked up to recorder of contractions. The height of the single isometric contraction of a uterine strip was measured. In principle, cumulative dosing was applied in the experiments, i.e. doses of standard (in the presence or absence of analogues) or of the analogue were added successively to the uterus in the organ bath in doubling concentrations and at 1 min intervals without the fluid being changed until the maximal response (the highest contraction) was obtained. The dose–response curves were constructed. Synthetic oxytocin was used as standard. Each new analogue was tested using uteri from 3 to 5 different animals; the values in Table 2 are averages ± SEM.

3.3. Vasopressor activity

The vasopressor test was performed using phenoxybenzamine-treated male rats [37] under urethane anesthesia. AVP was used as a standard. Changes of the arterial blood pressure were registered. Dose (single administration into vena femoralis)–response curves were constructed in the presence and absence of an antagonist. The antagonist was administered 1 min prior to the administration of the standard. Each analogue was tested in 3–5 independent experiments, the values in Table 2 are averages ± SEM.

With agonists, the activity was expressed in IU/mg, whereas with antagonists as pA_2. The pA_2 values represent the negative logarithm to the base 10 of the average molar concentration of an antagonist that reduces the response to 2*x* units of the agonist to the response to *x* units of the agonist. The volume of distribution in the in vivo experiments is arbitrarily taken as 67 ml/kg. The responses to standard doses of oxytocin or vasopressin were stable for several hours, without problems with tachyphylaxis. For details see Ref. [38].

3.4. Antidiuretic activity

Tests to assess the antidiuretic properties were conducted on conscious male rats using the modified Burn test [39,40]. In the standard manner with hydrated rats, the animals having fasted for 16 h were weighed and then given tap water through a stomach catheter. The water load was 4% of the body weight. Immediately after the water load, the tested substances (or physiological saline as control) were administered subcutaneously at doses of 0.001–100 nmol/kg. The rats were then placed in individual metabolic cages, and their urine was collected over a 5 h period. The time $t_{1/2}$ in which the rats excreted half the water load was determined and then plotted against the dose. As the dose–response curves were not parallel, such doses were chosen for calculating the compound's potency which yield $t_{1/2}$ equal to 60 min (the so-called threshold doses equal to the value of $t_{1/2}$ obtained with the physiological solution) and $t_{1/2}$ equal to 200 min. On each day of the experiment, 21 rats divided into 5 groups of 4 or 5 animals were administered different doses of different compounds; in an average

Table 2
Pharmacological properties of the new AVP analogues together with the values for AVP and some related analogues.

Analogue	Activity[f]					
	Uterotonic in vitro no Mg^{2+} IU/mg or pA_2	Pressor[g] IU/mg or pA_2	Antidiuretic[h]			
			IU/mg		% of activity of dDAVP	
			$t_{1/2}=60$	$t_{1/2}=200$	$t_{1/2}=60$	$t_{1/2}=200$
AVP[a]	17	412	450	450	10	0.125[e]
[Mpa^1,D-Arg^8]VP[a] (dDAVP)	1.5–5.1	~0.39	800–50 000	–	100	100[e]
[D-Arg^8]VP[a] (DAVP)	0.4	4.1	111–257	–	23.2	1.7[e]
[Mpa^1]AVP[a] (dAVP)	27–63	346–370	1300–1745	–	73.9	97.3[e]
[Val^4]AVP[a]	–	32	758	–	–	–
[Cpa^1]AVP[a]	$pA_2=8.15$	$pA_2=8.35$	0.033	–	–	–
[Acc^2]AVP[b]	$pA_2\sim5.6$	56.6	750–900	~9300	–	–
[Dip^2]AVP[c]	$pA_2=7.00\pm0.20$	0	450	9000	10	3.5
[Mpa^1,Dip^2]AVP[c]	$pA_2=7.20\pm0.08$	$pA_2=6.20$	450	45 000	10	18
[Aic^2]AVP[d]	$pA_2=7.27$	9.4 ± 2.8	~450	45 000	10	18
[Mpa^1,Dip^2,Val^4]AVP (**I**)	$1.7\pm0,2$	0	100	20 000	2.2	8
[Dip^2,D-Arg^8]VP (**II**)	$0.44\pm0,11$	0	30	3000	0.7	1.2
[Mpa^1,Dip^2,D-Arg^8]VP (**III**)	0.04 ± 0 01	0	30	20 000	0.7	8
[Cpa^1,Dip^2]AVP[c] (**IV**)	$pA_2=6.23\pm0.14$	0	<4.5	<4.5	<0.1	<0.1
[Mpa^1,Dip^2,Val^4,D-Arg^8]VP (**IV**)	$0.24\pm0,12$	0	100	4500	2.2	1.8
[D-Dip^2]AVP (**V**)	$pA_2=7.82\pm0.39$	0	1000	45 000	22	18
[Mpa^1,D-Dip^2]AVP (**VI**)	$pA_2=7.80\pm0.14$	0	4500	200 000	100	~80
[Cpa^1,D-Dip^2]AVP (**VII**)	$pA_2=6.83\pm0.32$	$pA_2=7.4$	4.5	400	0.1	0.5
[Mpa^1,D-Dip^2,Val^4]AVP (**VIII**)	7.6 ± 4.3	$pA_2<5.8$	4500	450 000	100	~150
[D-Dip^2,D-Arg^8]VP (**IX**)	$pA_2=7.83\pm0.08$	0	1000	20 000	22	8
[Mpa^1,D-Dip^2,D-Arg^8]VP (**X**)	$pA_2=7.79\pm0.09$	$pA_2=5.8$	9000	225 000	200	~100
[Mpa^1,D-Dip^2,Val^4,D-Arg^8]VP (**XI**)	$pA_2=7.78\pm0.08$	$pA_2<5.8$	9000	450 000	200	~150

[a] Values taken from Ref. [17].
[b] Values taken from Ref. [20].
[c] Values taken from Ref. [21]; in that paper 3,3-diphenylalanine was abbreviated as Dpa.
[d] Values taken from Ref. [41].
[e] Values taken from Ref. [45].
[f] The values are given as averages ± SEM, $n=3$–5.
[g] 0 means no activity up to the dose of 0.15 mg/kg of experimental animal.
[h] Activity was estimated on the threshold level of activity 60 min and on the level of the 200 min activity; activity of AVP taken as 450 IU/mg at both levels.

each compound was tested in 3–5 different doses, each dose being tested in 2 or 3 independent experiments (different days, different rats). The results were thus expressed in IU/mg in comparison to AVP (the value 450 IU/mg was taken for AVP for both $t_{1/2}$ 60 min and $t_{1/2}$ 200 min). All peptides showed some degree of antidiuretic activity and no peptide enhanced the quantity of excreted urine or speed-up the excretion in the course of any experiment (in comparison to controls). We have thus not expected a diuretic effect and not performed the diuretic test using non-hydrated rats.

4. Results

The 11 new analogues of AVP (**I**–**XI**) were synthesized by Boc strategy, purified by HPLC and characterized. The purity of all the analogues was higher than 98%. Physicochemical properties of the new peptides and their pharmacological data are presented in Tables 1 and 2, respectively.

The activities of the analogues were determined in the in vitro rat uterotonic test in the absence of magnesium ions, in the rat pressor test, and in the antidiuretic assay using conscious rats as described in the experimental section. The comparison of the antidiuretic activities of the new analogues with those published previously in the literature is complicated by the fact that different methods were used for the activity determination and that the dose–response curves of the analogues and that of standard AVP have different slopes (Fig. 2). It is therefore necessary to give two values of potency, the first one resulting from comparison of the threshold doses of AVP with those of the analogues (antidiuresis time $t_{1/2}$ 60 min) and the second one originating from comparison of doses giving an antidiuresis time of 200 min. The antidiuresis time ($t_{1/2}$) corresponds to

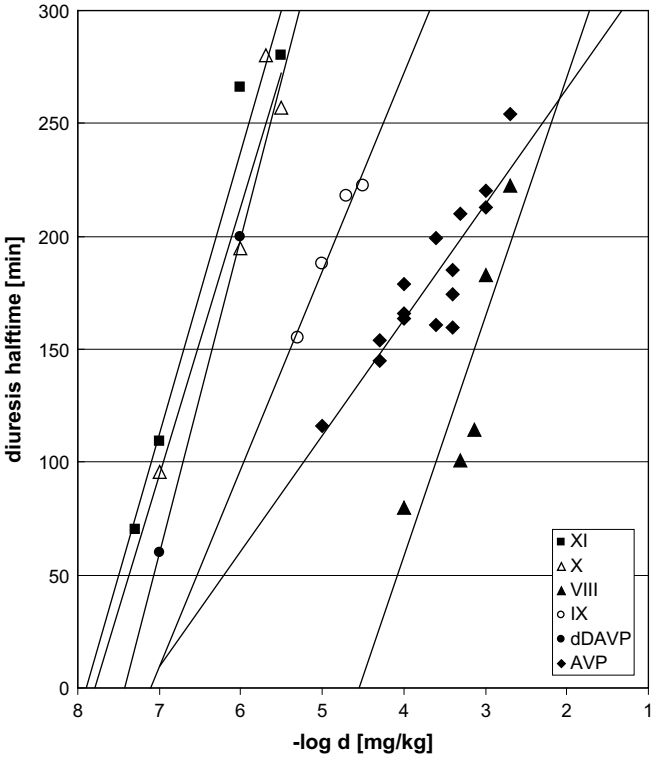

Fig. 2. Dose–response curves of the antidiuretic effect of analogues deamino[D-Dip^2, Val^4,D-Arg^8]VP (**XI**, ■), deamino[D-Dip^2,D-Arg^8]VP (**X**, △), [D-Dip^2,D-Arg^8]VP (**IX**, ○), [Cpa^1,D-Dip^2]AVP (**VIII**, ▲) and standards deamino[D-Arg^8]VP (dDAVP, •) and AVP (◆).

the time in which the rat excretes half of the water load. For AVP, the activity has arbitrarily been set to 450 IU/mg for both responses.

Peptides **I**–**IV** with Dip at position 2 have antidiuretic activity on the threshold level 4–15 times lower than that of AVP, at the 200-min level they are however 7–45 fold more effective than AVP. Analogues **V**, **VI**, **VIII**–**XI** with D-Dip at position 2 exhibit much higher activity than AVP already at the level of $t_{1/2}$ 60 min (2–20 times), and as their dose–response curves are also much steeper in comparison with that of AVP (comparable to dDAVP), the activity at the $t_{1/2}$ level of 200 min is much higher than that of AVP (up to 1000 times). Analogue **VII** showed only low antidiuretic activity – Cpa^1 modification decreased both antioxytocic and antidiuretic activities.

Peptides **I**–**VI** and **IX** did not exhibit pressor or antipressor activities while the remaining analogues were either moderate (**VII**) or week (**VIII**, **X**, **XI**) pressor antagonists. Analogues **I**–**IV** and **VIII** showed low agonistic activity in the uterotonic test in vitro, while peptides **V**–**VI** and **IX**–**XI** exhibited high antioxytocic potency ($pA_2 = 7.78$–7.83), only analogue **VII** displayed one order of magnitude lower antioxytocic activity ($pA_2 = 6.83$).

5. Discussion

We have previously demonstrated significant impact of steric restrictions and bulkiness of the substituents in the N-terminal part of the AVP and OT molecules on pharmacological properties of the resulting analogues. Our studies included among others the modification of AVP and some of its analogues with Acc and Apc, which resulted in compounds having high antidiuretic potency, decreased pressor activity and either no activity or low oxytocin antagonizing activity in the uterotonic in vitro tests [24–26]. Recently we presented the synthesis and some pharmacological properties of the AVP analogues substituted in position 2 with 2-aminoindane-2-carboxylic acid (Aic) [41]. This modification resulted in similar changes of pharmacological properties as that which caused modification with Apc and Acc. Enlargement of the cyclic side chain and restoring its aromatic character, placing the aromatic ring closer to the backbone in comparison to Tyr, led either to preservation or a slight decrease in antidiuretic activity as calculated from the threshold doses, as well as enhancement of activity when comparing doses eliciting a 200 min antidiuresis. One of the peptides, namely [Mpa^1,Aic^2,Val^4,D-Arg^8]VP, had an antidiuretic activity similar to that of dDAVP, thus being one of the most potent antidiuretic peptides reported to date [41]. These, in our opinion interesting findings, promoted us to further investigate the impact of reducing the conformational freedom of the N-terminal part of the AVP analogues by placing in position 2 other bulky residue 3,3-diphenyl-L-alanine (Dip). The results were encouraging [27]. Single modification of the AVP or dAVP molecules, i.e. substitution of Tyr^2 with Dip resulted in peptides with strikingly different pharmacological properties in comparison with the parent compounds (Table 2). The two analogues were moderately potent uterotonic antagonists, displayed no activity or low antagonism in the pressor test while their antidiuretic activity was preserved and prolonged.

In this study we have introduced the same non-proteinogenic amino acid Dip into other 4 analogues. We have also introduced its D-enantiomer (D-Dip) into position 2 of the same 7 analogues. The results presented in Table 2 show that antidiuretic potency of the analogues modified at position 2 with Dip and additionally having different combinations of changes at positions 1 and 4 is lower than that of AVP as calculated on the basis of the threshold doses. On the other hand, the peptides exhibit significantly prolonged activity. The Dip modification itself results in prolongation of the effect. Mpa^1 and Val^4 substitutions commonly used to increase antidiuretic potency and D-Arg introduction in position 8 which increases the selectivity [42,43], did not further increase the antidiuretic activity substantially. All the Dip^2 substituted analogues are very selective as they do not show any pressor activity and exhibit either very low or negligible uterotonic activity.

As far as analogues **V**–**XI** modified in position 2 with D-Dip are concerned, it can be seen that all the new peptides, with the exception of [Cpa^1,D-Dip^2]AVP, are very potent and selective agonists of V_2 receptors. Their activity is about 2–20 fold higher than that of AVP as calculated on the basis of the threshold doses. They are, however, 44–1000 fold more active than AVP when comparing doses giving the 200 min antidiuresis. This means that up to 1000 times less of the compound is necessary to evoke the same effect ($t_{1/2} = 200$ min). Of course we do not know whether the prolongation of the activity is due to better binding to the receptor, or better signal transformation or slower elimination from the organism.

It is very interesting to note that only a single modification of position 2 with either Dip or D-Dip changes dramatically the properties of the parent hormone: preservation of the antidiuretic activity and its prolongation, transformation of uterotonic into antiuterotonic properties and elimination of any effect on blood pressure. It is also worth noting that the effect is more pronounced with the D-Dip than with Dip (compare the activities of analogue **V** with that of its Dip counterpart). In the D-series, the impact of deamination of position 1 (Mpa^1) was as expected – the antidiuretic potency of the resulting compound **VI** was enhanced. Additional substitution of this analogue in position 4 with Val resulted in further prolongation of the activity (peptide **VIII**) and appearance of a very weak antipressor activity.

In regard to the uterotonic test, the new peptides **V**–**XI** have high antioxytocic potency with the exception of analogue **VII** which displays weak antioxytocic properties and of analogue **VIII** which exhibits low agonistic activity. High antioxytocic activity of most of our analogues is very interesting, especially that of peptide **V** that differs from AVP in position 2 only. It is also worth emphasizing that analogue **VII** having a bulky and lipophilic substituent in position 1 is a relatively weak antagonist of oxytocin in the uterus in vitro test, which is in contrast with the previous knowledge [42,43]. Another surprising finding is the low uterotonic agonistic activity of peptide **VIII** which differs from the potent antagonist of oxytocin (peptide **VI**) by the presence of Val at position 4 only. The Val^4 modification was thought to increase antidiuretic activity. In the case of dAVP [21] introduction of Val in position 4 also enhanced the uterotonic activity. These observations support the hypothesis that in the case of analogues having conformational restriction in the N-terminal part of the molecule accumulation of modifications does not result in enhancement of the desired activity. Single modifications leading to the same effect, e.g. enhancement of antidiuretic activity, may not necessarily have an additive effect if introduced together.

As far as the pressor test is concerned, it is clear that in comparison to Tyr^2 the D-Dip^2 analogues are either devoid of the pressor activity or become converted into very weak vasopressin antagonists. In the case of [Cpa^1]AVP, both the Dip^2 [27] and D-Dip^2 modifications (analogue **VII**) strongly reduced its antipressor potency. Probably two bulky substituents in the vicinity of one another (positions 1 and 2) cannot be tolerated by the receptors.

Usually, replacement of Tyr^2 with proteinogenic amino acids leads to the decrease of all biological activities. An exception presents [Phe^2]AVP and [D-Tyr^2]AVP and their deamino analogues [21]. They have decreased uterotonic activity, slightly reduced pressor activity and preserved or enhanced antidiuretic activity. D-Dip with its 2 bulky aromatic phenyl groups may disturb the so called stacking of aromatic side chains in positions 2 and 3 and this way reduce markedly the pressor effect. The so called "stacking" is not important for the antidiuretic activity.

In general, the presence of D-Dip at position 2, in comparison with the L-enantiomer, more distinctly enhances antidiuretic activity of the peptides and in the case of pressor activity it triggers a weak vasopressin V_{1a} antagonism.

On the basic of these results, we are undertaking further investigations on our Dip[2] and D-Dip[2] modified AVP analogues to determine binding affinity to V_{1a}, V_{1b}, V_2 and OT receptors. Parallely we are performing the structural analysis and molecular modeling of the peptides that may help to reveal processes underlying these interesting findings.

6. Conclusion

In summary, our studies have shown once more that the modification of position 2 which changes conformation of the N-terminal part of the analogue has a dramatic impact on the pharmacological profile of the compound (in this case it resulted in a high and prolonged V_2 agonistic activity). This finding supports our previous results showing that single substitution with some bulky residues may result in quite potent antagonists of OT receptors [44]. Such substitution is clearly disadvantageous when applied to antagonists of V_{1a} receptors, as has been documented in our previous paper [25]. It should be emphasized that four of the new peptides ([Mpa[1],D-Dip[2]]AVP, [Mpa[1],D-Dip[2],Val[4]]AVP, [Mpa[1],D-Dip[2],D-Arg[8]]VP, [Mpa[1],D-Dip[2],Val[4],D-Arg[8]]VP) are exceptionally potent antidiuretic agonists with significantly prolonged activities, which makes them good candidates for studying the physiological role of AVP and potential therapeutic agents.

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